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REGULATION OF PHOSPHOFRUCTOKINASE ACTIVITY BY GLUCAGON IN ISOLATED RAT HEPATOCYTES

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SUMMARY

Glucagon addition to isolated hepatocytes from fed rats resulted in an inhibition of the activity of phosphofructokinase measured in extracts of the cells. Glucagon caused a shift in the fructose 6-phosphate concentration curve to the right resulting in an increase in the $K_{0.5}$ for F6P from 0.09 mM to 0.31 mM. No effect of glucagon was seen when the enzyme was assayed with saturating concentrations of fructose 6-phosphate or in the presence of 1 mM AMP. The effect of glucagon was seen within minutes and the concentration of hormone giving half-maximal inhibition was 0.2 nM. This effect of glucagon on phosphofructokinase activity may contribute to the effect of glucagon on substrate cycling at the fructose 6-phosphate-fructose bisphosphate level.

INTRODUCTION

We have put forth the hypothesis that hormones acutely affect carbon flux in the hepatic gluconeogenic pathway by modulating the activity of several enzymes involved in the substrate cycles between pyruvate and phosphoenolpyruvate and fructose bisphosphate and fructose 6-phosphate (1). The firmest support for this hypothesis comes from studies on the hormonal regulation of L-type pyruvate kinase (1-4). With regard to the fructose bisphosphate-fructose 6-phosphate substrate cycle, a number of studies, using isotopic methods, have indicated that glucagon and catecholamines suppress carbon flux at the phosphofructokinase step and enhance flux through fructose bisphosphatase (5-7). Both fructose bisphosphatase and phosphofructokinase have been shown to undergo *in vitro* phosphorylation by protein kinases with concomitant activity changes (8-10). However, few reports on the acute effect of hormones on the activities of these enzymes have appeared (11,12,13). In this paper, we report that glucagon addition to isolated hepatocytes results in an inhi-

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bition of phosphofructokinase activity which is characterized by an apparent decrease in the enzyme's affinity for its substrate fructose 6-phosphate.

METHODS

Preparation and Incubation of Isolated Rat Hepatocytes. Isolated rat hepatocytes were prepared from fed rats as previously described (14). The cells were suspended in Krebs-Henseleit buffer that contained 0.5% bacitracin and incubated without any additions for 20 min at 37°. Five ml aliquots (100 µg DNA/ml) of the suspension were incubated with various additions and for various times as indicated in the figure legends. Cells were centrifuged rapidly (20 sec), resuspended in 1.5 ml of homogenizing buffer that contained 50 mM Tris, pH 8.0, 2.5 mM dithiothreitol, 0.1 mM EDTA, and 50 mM NaF, and homogenized for 90 sec (30 sec, 3 times) with an Ultraturrax homogenizer (Tekmar Co., Cincinnati, Ohio) at full speed. The homogenate was centrifuged at 27,000 x g for 30 min. The supernatant fraction was then heated at 55° for 3 min and the denatured protein sedimented at 27,000 x g for 10 min. The heated extract was assayed for phosphofructokinase activity.

Phosphofructokinase Assay. The assay mixture contained in a final volume of 1 ml: 50 mM Tris-HCl (pH 7), 1 mM EDTA, 6 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.165 mM NADH, 1 mM ATP, aldolase (0.4 unit), triose phosphate isomerase (2.4 units), and α-glycero-P-dehydrogenase (0.4 unit) and various concentrations of fructose 6-phosphate. The hepatocyte extract was preincubated with the assay mixture minus fructose 6-phosphate for 4 min at 30° and then the reaction initiated with fructose 6-phosphate. The decrease in absorbance at 340 nm was measured at 30° with a Beckman Model 24 Recording Spectrophotometer (15). One unit of phosphofructokinase is defined as the amount of enzyme that catalyzed the conversion of 1 µmole of fructose 6-phosphate to fructose bisphosphate per minute at 30° under the conditions of the assay.

When measuring phosphofructokinase activity in crude, unheated hepatocyte extracts there was a high rate of endogenous oxidation of NADH. This oxidation occurred in the absence of ATP, fructose 6-phosphate, and the coupling enzymes. Assay of the enzyme as a function of fructose 6-phosphate was difficult since the endogenous rate of NADH oxidation was equal to the rate of the phosphofructokinase reaction measured under maximal velocity conditions. This endogenous rate of NADH oxidation could be eliminated by heating the hepatocyte extract at 55° for 3 min. Such heat treatment had no effect on the total activity of phosphofructokinase but permitted the accurate determination of the $K_{0.5}$ for F6P for the enzyme. This heat step is commonly used in the purification of mammalian phosphofructokinases (15).

The activity of phosphofructokinase is expressed as the ratio of activity measured at a submaximal substrate concentration to that measured at 4 mM fructose 6-phosphate. Under our assay conditions, maximum activity was observed with 4 mM and hormones did not affect the maximum activity under any circumstances. All experiments were repeated at least three times. The weight of hepatocytes was estimated from the DNA content as previously described (16).

RESULTS

Characterization of the Glucagon-Induced Inhibition of Hepatocyte Phosphofructokinase Activity. Preliminary experiments in which extracts were

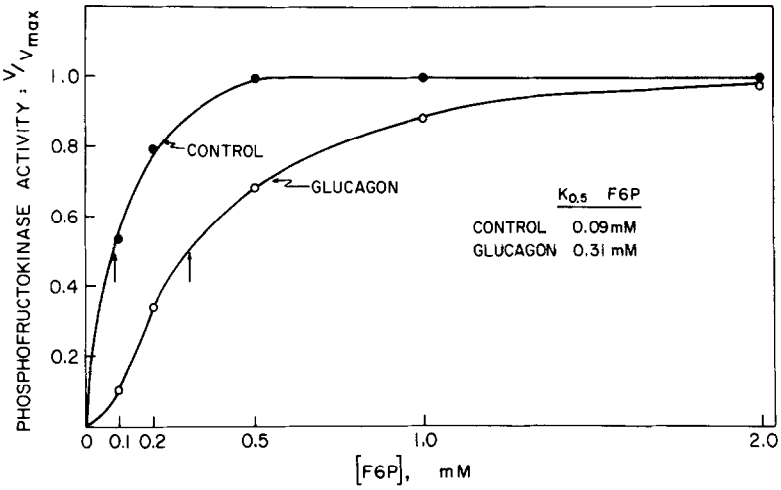

Figure 1. Effect of Glucagon on Hepatocytes Phosphofructokinase Activity. Isolated hepatocytes (200 mg/5 ml) were prepared as described in Methods and incubated in the presence and absence of 10 nM glucagon for 10 min. Cells were sedimented and homogenized as described in Methods and assayed for phosphofructokinase activity as a function of fructose 6-phosphate concentration. The activity of the enzyme measured at 4 mM fructose 6-phosphate and 1 mM ATP was control: 2.4 Units/gm of hepatocytes; glucagon: 2.3 Units/gm of hepatocytes. Phosphofructokinase activity is expressed as V/V_{max} where V is the velocity of the reaction at any particular substrate concentration and V_{max} is the velocity with 4 mM fructose 6-phosphate.

prepared from cells incubated with and without 10 nM glucagon revealed no effect of the hormone on phosphofructokinase activity when the enzyme was measured under V_{max} conditions (4 mM F6P). Since glucagon inactivation of pyruvate kinase was only observed at subsaturating concentrations of substrate (1), we investigated whether a similar situation existed for phosphofructokinase by determining the activity of the enzyme as a function of the fructose-6-phosphate concentration (Fig. 1). In the absence of hormone, phosphofructokinase exhibited sigmoidal kinetics and the $K_{0.5}$ for F6P was 0.09 mM. In the presence of glucagon, the enzyme showed greater sigmoidicity and the curve shifted to the right. The $K_{0.5}$ for F6P increased to 0.31 mM. This apparent increase in the $K_{0.5}$ for F6P caused by glucagon is very similar to the inhibitory effect of the hormone on pyruvate kinase activity, which is also characterized by an increase in the $K_{0.5}$ for that enzymes' substrate (1-4).

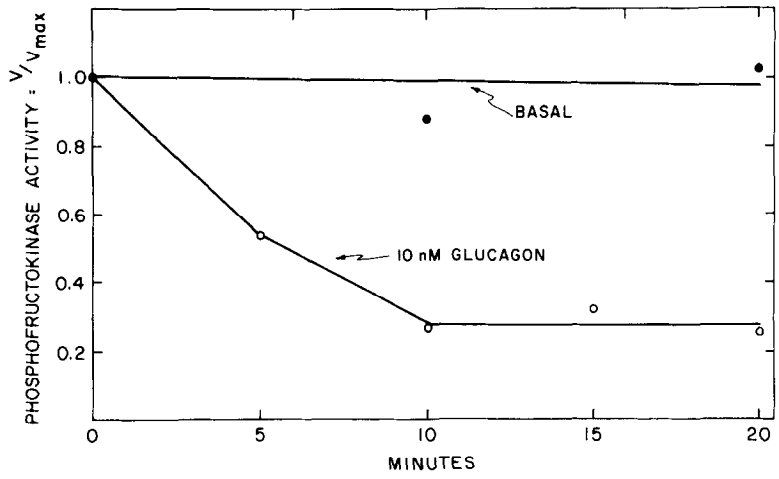

Figure 2. Time Course of the Effect of Glucagon on Hepatocyte Phosphofructokinase Activity. Hepatocytes (200 mg/5 ml) were prepared and incubated as described in the Methods. Aliquots (5 ml) were removed and hepatocyte extracts prepared. Phosphofructokinase activity was measured at 0.2 mM and 4.0 mM fructose 6-phosphate and V/V_{max} represents the ratio of activities at these two substrate concentrations. The glucagon concentration was 10 nM.

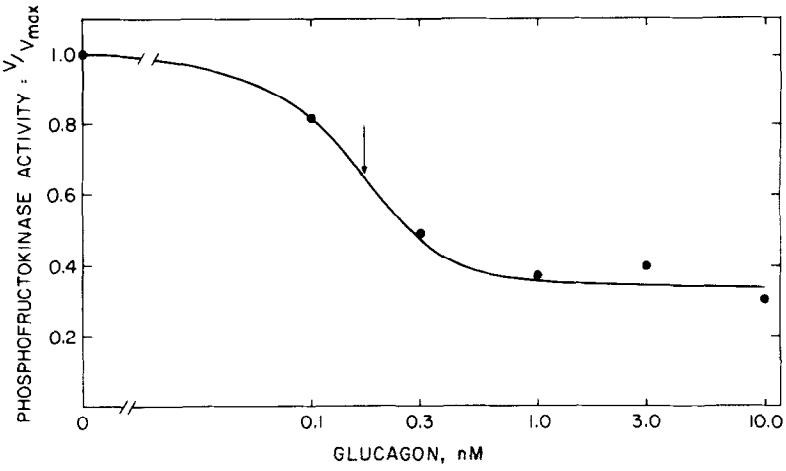

Figure 3. The Effect of Glucagon Concentration on Phosphofructokinase Activity in Isolated Hepatocytes from Fed Rats. Hepatocytes (200 mg/5 ml) were incubated with various concentrations of glucagon for 10 min. The cells were sedimented and hepatocyte extracts prepared as described in Methods. Phosphofructokinase activity is expressed as the ratio of activity (V/V_{max}) at 0.2 mM fructose 6-phosphate to that at 4 mM fructose 6-phosphate. The vertical arrow indicates the concentration of hormone giving half maximal inactivation of the enzyme.

Liver phosphofructokinase is regulated by a multitude of metabolites (17, 18). It seemed reasonable to assume that the glucagon-induced inhibition of the enzyme could be either accentuated or attenuated by measuring the enzyme

in the presence of various effectors particularly the adenine nucleotides. ATP is a potent allosteric inhibitor of phosphofructokinase and when phosphofructokinase from extracts of hepatocytes treated with and without glucagon was assayed with 3 mM ATP instead of 1 mM, the enzyme was inhibited ($K_{0.5}$ F6P: Control, 0.29; Glucagon, 0.50), but the effect of glucagon was actually less than when the enzyme was assayed with 1 mM ATP . Glucagon shifted the F6P concentration curve to the right but did not alter the maximal velocity (data not shown). When the enzyme was assayed in the presence of 1 mM AMP, a potent activator of the enzyme, the effect of glucagon was not seen because the enzyme was completely activated in extracts from both control and glucagon treated cells ($K_{0.5}$ F6P: Control, 0.02 mM; Glucagon, 0.02 mM). Inactivation of pyruvate kinase by glucagon is also not observed when that enzyme is assayed in the presence of its allosteric activator, fructose bisphosphate (1). Figure 2 illustrates the time course of inactivation of phosphofructokinase in extracts of hepatocytes incubated with 10 nM glucagon. Maximal inactivation (70% inhibition) was not obtained until 10 min and then it was sustained for at least another 10 min.

The inactivation of phosphofructokinase by various concentration of glucagon is shown in Figure 3. The half-maximally effective concentration of glucagon was about 0.2 nM. This concentration of hormone is similar to that which is necessary for half-maximal stimulation of gluconeogenesis in hepatocytes (1) as well as for half-maximal inactivation of pyruvate kinase (1-3).

Effect of Other Hormones on Phosphofructokinase Activity.

Several studies have demonstrated an effect of catecholamines to inhibit carbon flux through phosphofructokinase in isolated hepatocytes (6,7). Table I shows the effect of catecholamines as well as that of insulin on hepatocyte extract phosphofructokinase. Epinephrine (10 μM) had a small inhibitory effect on hepatocyte extract phosphofructokinase activity whereas 1 μM phenylephrine had no effect. This concentration of phenylephrine has no effect on hepatocyte cyclic AMP levels or on the activation of cyclic AMP-

Table I

Phosphofructokinase Activity in Extracts of Hepatocytes Incubated with Various Hormones

Hormone Added	V/V_{max}
None	0.78
Glucagon, 10 nM	0.36
Glucagon, 0.3 nM	0.48
Epinephrine, 10 μM	0.65
Phenylephrine, 1 μM	0.79
Insulin, 10 nM	0.69
Glucagon, 0.3 nM + Insulin, 10 nM	0.41

Hepatocytes from fed rats were incubated with various hormones for 10 min and extracts prepared as described in Methods. Phosphofructokinase activity is expressed as the ratio (V/V_{max}) of activity at 0.2 mM fructose-6-phosphate to that at 4 mM substrate. None of the hormones affected activity at 4 mM fructose-6-phosphate.

dependent protein kinase (19). Insulin (10 nM) at concentrations which lower glucagon-stimulated cyclic AMP levels (20) and which oppose the action of glucagon to inhibit pyruvate kinase (4), had little effect on phosphofructokinase activity alone or when added with a submaximal concentration of glucagon (0.3 nM).

DISCUSSION

This report clearly demonstrates that glucagon addition to an isolated liver system results in inhibition of phosphofructokinase activity. Taunton et al. (11) demonstrated that glucagon administration to intact rats resulted in an inhibition of phosphofructokinase activity measured under V_{max} conditions. The effect of glucagon on phosphofructokinase activity in hepatocytes is similar to the effect of the hormone on pyruvate kinase activity in that the $K_{0.5}$ for the substrate is increased. The effect of glucagon is seen best when either enzyme is assayed at substrate concentrations which are in the range of their physiological concentration in liver (Fig. 1; ref. 2).

In view of the demonstration that glucagon causes inactivation of pyruvate kinase *in vivo* and in isolated liver systems by a cyclic AMP-dependent phosphorylation mechanism (1-4,21), it is attractive to postulate a similar mechanism for the regulation of phosphofructokinase. However, there is no evidence that phosphorylation of hepatic phosphofructokinase can be catalyzed

by cyclic AMP-dependent protein kinase or that such phosphorylation alters the activity of the enzyme. Phosphofructokinase in crude liver extracts can be phosphorylated by a cyclic AMP-independent protein kinase with a concomitant increase in enzyme activation (9,10).

Hormones may also affect enzyme activity by influencing the intracellular level of various allosteric effectors of the enzyme. Epinephrine administration to intact rats results in transitions between labile and stable forms of phosphofructokinase in rat diaphragm (22). The amount of phosphofructokinase found in the extracts depended on the concentration of various effectors present in the extracts. Glucagon has no effect on the intracellular levels of the various adenine nucleotide effectors of the enzyme (5,12) and has been reported to depress the level of citrate, an inhibitor of the enzyme (23). Glucagon lowers the level of fructose bisphosphate (1) a potent activator of phosphofructokinase (24-26). Experiments are in progress to determine whether glucagon acts by means of phosphorylation-dephosphorylation reactions and/or via changes in the level of allosteric effectors. Whatever the mechanism, this inhibition probably contributes to the glucagon-induced suppression of phosphofructokinase flux in intact hepatocytes (1,5-7).

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